**Appendix 4**

Research Protocol 936-9212

Determination Of The Effect Of The Listerine® Essential Oils On The Anticaries Activity Of A Fluoride Mouthrinse Using A Rat Caries Model

CONFIDENTIAL PROTOCOL FOR WARNER-LAMBERT
ORAL CARE DEVELOPMENT

# DETERMINATION OF THE EFFECT OF THE LISTERINE® ESSENTIAL OILS ON THE ANTICARIES ACTIVITY OF A FLUORIDE MOUTHRINSE USING A RAT CARIES MODEL

**STUDY #936-9212**

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## Tentative Timetable

| | |
|---|---|
| Initiation of Treatment Phase | July 7, 2000 |
| Termination of Treatment Phase | August 4, 2000 |
| Scoring Completed | September 8, 2000 |
| Final Report | September 22, 2000 |

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## 1. STUDY OBJECTIVE

The objective of this study is to determine whether the fixed combination of Listerine essential oils affects the anticaries efficacy of a fluoride mouthrinse containing 0.022% NaF (100 ppm fluoride ion).

## 2. BRIEF DESCRIPTION OF EXPERIMENTAL RATIONALE AND METHOD

### 2.1. Rationale

The FDA final monograph on OTC Anticaries Drug Products (21 CFR, § 355.10(a)(3)(iii)) accepts a sodium fluoride 0.022% aqueous solution with a pH of approximately 7 as safe and effective for preventing dental caries. The fluoride mouthrinse to be evaluated in this study is a 0.022% sodium fluoride solution with a pH of 4.2 and also containing the fixed combination of essential oils found in Listerine® Antiseptic mouthrinse. This study will use a rat caries model to confirm that **the test fluoride mouthrinse** is an effective anticaries treatment, and to assess whether the Listerine ingredients influence the anticaries activity of the mouthrinse. The study will include five cells, an FDA monograph-compliant neutral sodium fluoride mouthrinse containing 0.022% NaF (100 ppm fluoride ion) as **a positive control**, sterile distilled water as **a negative control**, a fluoride-free Listerine® Antiseptic mouthrinse as **treatment control I,** and an acidulated (pH=4.2) fluoride mouthrinse containing 0.022% NaF as **treatment control II**.

### 2.2. Study Method

The animal caries model to be used is a modification of FDA method #37 (Docket No. 80N-0042). Modifications include the use of Sprague Dawley rats (instead of Wistar), the use of the NIH 2000 cariogenic diet (instead of diet #469), the use of a 5-week treatment duration (instead of 3 weeks), and the use of the Keyes method of scoring caries (instead of the HMA method)[see Appendix I]. Experimental procedures will be conducted according to the GLP regulations (12CFR Part 58).

The model entails inoculating young Sprague Dawley rats with a cariogenic strain of *Streptococcus sobrinus*, maintaining the animals on a cariogenic diet, and treating the animals twice daily (once daily on weekends) with the assigned mouthrinse for five weeks, after which the animals are sacrificed and scored for caries. Each test cell will contain 35 animals.

## 3. JUSTIFICATION FOR ANIMAL USE AND CELL SIZE

A battery of laboratory tests and a rat caries study have been generally accepted as a means of documenting the anticaries effectiveness of certain fluoride-containing products in lieu of long-term clinical trials in children. The rat caries studies have typically included a negative control group, an experimental group treated with the new, test formulation, and a positive control group treated with a similar fluoride product whose caries-preventive effectiveness had been previously demonstrated in a controlled human clinical caries trial. The recognized reliability and biological relevance of the rat caries model are the basis for choosing this model to answer the questions of effectiveness and non-interference to be addressed in this study.

Results from previous studies conducted by the Principal Investigator indicate that 35 animals per treatment group at study initiation are appropriate to achieve the study objectives. Statistical power is further enhanced by balancing across litters and the use of litter as a second factor in a two-way

analysis of variance.

## 4. EXPERIMENTAL DESIGN

This observer-blind, placebo-controlled study will employ a randomized block design, with complete balancing of treatments across litters.

## 5. TEST MATERIALS

The test materials to be used in this study are:

| # | Function of the Cell | Product Name | Product # |
|---|---|---|---|
| 1 | Negative Control | Water | W10859-003 |
| 2 | Test Fluoride Mouthrinse | Listerine with Fluoride, pH =4.2 | W2194-471 |
| 3 | Treatment Control I | FreshBurst Listerine, pH=4.2 | W2194-396 |
| 4 | Treatment Control II | NaF mouthrinse, pH=4.2 | W2194-472 |
| 5 | Positive Control | Neutral NaF mouthrinse | W2194-473 |

The test mouthrinse, positive control, and the treatment controls will be produced in the CHR&D Oral Care Development pilot plant in accordance with GMP's. The negative control (sterile water) will be purchased from a commercial source. The test site will store all products at room temperature. All solutions will be packaged in coded plastic bottles. To further reduce bias during treatment and scoring, treatment identity will be withheld from personnel administering treatments and performing analyses. All bottles (including empties) will be kept and returned to the sponsor at study completion.

## 6. EXPERIMENTAL MATERIALS AND METHODS

### 6.1. Animals

6.1.1. Type of Animals:

Weanling female Sprague Dawley rats will be received as 14-day-old pups with dams. All the rats will be weighed upon receiving and will be weighed weekly through the study.

6.1.2. Pathogen Screening:

Dams will be screened for mutans streptococci and antibody for SDV virus; positive dams and their litters will be rejected from the study.

6.1.3. Animal source:

Charles River Inc. (Kingston (K93 Facility), NY).

6.1.4. Housing:

The litters will be maintained in large solid bottom box cages with their dams until the pups are weaned at 20 days of age. Dams and pups will receive rat chow and deionized water with 10%

sucrose until the pups are weaned at 20 days of age, after which the pups will be assigned to groups and housed in pairs in cages which have been cleaned and sanitized prior to use. The cages will be arranged so that all animals of each group are together, and the cages will be labeled with group code. The rats will be transferred to clean suspended wire-bottomed cages midway through the treatment period (i.e. after 2.5 weeks of treatment).

6.1.5. Identification and Stratification:

When the animals are assigned to treatment groups at 20 days old, they will be given unique numbers and placed in cages in pairs in numerical order; the numerically first rat will have no ear clip, while the second rat will be marked with an ear clip in each ear. For example, Red 1 and Red 2 will be caged together, and Red 2 will be earclipped. Animals will be assigned to groups in such a manner that groups are balanced with respect to litter, weight and gender. Each group will contain 35 animals (see section 3). Records will be kept of littermates.

6.1.6. Random Assignment of Treatments:

After animals are assigned to groups, treatments will be assigned to each group according to a random code (Appendix II) to be held separately by a representative designated by the Study Director. This representative will be responsible for ensuring that product tubes are properly coded, and that products are properly administered to each treatment group.

6.1.7. Animal Care:

Animals will be provided with diet NIH 2000 and deionized water containing 5% sucrose *ad libitum* throughout the test period. Absorbent cage boards, as well as food and water bottles will be changed in a schedule consistent with good animal hygiene. The animals will be observed daily for any sign of health problems. Animals will be weighed weekly from the start of treatment.

6.1.8. Room Environment:

The animals will be housed in an AAALAC-accredited facility. Room temperature will be maintained at 72°F (±6°F) with 10-15 air changes per hour and a 12-hour light cycle.

6.1.9. Cariogenic Microflora Inoculation:

All pups and dams will be provided with Rat chow and deionized water with 10% sucrose until the pups are 20-22 days old. The diet will be changed to NIH 2000 when the pups are 20-22 days old, at which time they will be inoculated orally with a culture of streptomycin-resistant *Streptococcus sobrinus* 6715.

6.1.10. *Streptococcus sobrinus* Recovery:

At study termination, one half of the lower jaw of each rat will be aseptically dissected and placed in 3-5 ml of sterile distilled water and sonicated to dislodge adherent bacteria. The resulting suspension will be used to inoculate blood agar and Mitis Salivarius agar with streptomycin to estimate total cultivable flora and populations of *S. sobrinus*.

**6.2. Treatment procedures**

6.2.1. Treatment Initiation:

The treatment phase will begin when pups are 22 days old. The rats will be weighed upon receiving

and will be weighed weekly throughout the study.

6.2.2. Duration of Treatment:

Animals will treated for five weeks.

6.2.3. Mouthrinse Preparation and Labeling:

The investigator's staff will assign color codes to each mouthrinse, recording the product-color assignment in their laboratory records; each bottle of each product will be labeled with colored lab tape corresponding to the color code. Fresh aliquots of each treatment mouthrinse will be dispensed into plastic test tubes or beakers for each treatment.

6.2.4. Mouthrinse Treatment:

A cotton-tipped applicator will be dipped into the mouthrinse (for 2 seconds) and applied to one half of the rat's mouth in such a way that the sides of the applicator come into contact with both the mandibular and maxillary molars. The treatment will be accomplished using a rolling motion of the sides of the applicator over the mandibular and maxillary molar teeth for 15 seconds. The applicator will be dipped into the rinse for a second time (for 2 seconds) and the other side of the rat's mouth will be similarly treated for 15 seconds. A new applicator will be used for each animal, although a single beaker of mouthrinse will be used for the entire treatment group.

6.2.5. Schedule of Treatment Applications:

Treatments will be administered twice daily, five days per week, and once daily on weekends. The first of the twice-daily treatments will begin at approximately the same time each day (i.e., at 9 am); the second treatment will begin no earlier than five hours after the first treatment (approximately 2:30 p.m.). Weekend-treatments will be administered at mid-day (approximately 11:30 a.m.).

Food and water will be withheld from the rats for approximately 30 minutes following each treatment to minimize dilution effects occasioned by eating or drinking immediately following mouthrinse application.

6.2.6. Storage of Material:

Treatment materials will be stored at room temperature. All materials will be returned to sponsor at study completion.

### 6.3. Termination of Treatment and Caries Scoring

6.3.1. Final Observation and Examination:

Immediately prior to termination, all animals will be examined for any visual signs of ill health or pathology, individually weighed and swabbed (section 6.1.8) to confirm *S. sobrinus* implantation.

6.3.2. Animal Euthanization and Post-mortem Procedures:

All animals will be euthanized by carbon dioxide inhalation, an AVA-accepted euthanization procedure. Code numbers will be assigned to each animal to assure blindness to treatment group during scoring. The heads will be removed, placed in individual jars along with a tag carrying the code number, and frozen until they can be autoclaved for 3 minutes at 10 psi to loosen soft tissue. If autoclaving is not possible, the heads may alternatively be heated by microwaving. The hemijaws

will be surgically removed from the heads and freed of soft tissue.

6.3.3. Staining and Caries Scoring:

The four hemijaws of each rat will be placed into the beakers with the code number labels. The jaws will be scored for smooth surface caries using the Keyes method, then stained (to enhance visualization of carious lesions) by overnight (≈18 hr) soaking in a murexide solution (0.24 g murexide in 300 ml DI water and 700 ml of ethanol). After staining, the jaws will be rinsed and allowed to air dry. The hemijaws will be sectioned, and microscopically examined for sulcal and interproximal caries using the Keyes method (Appendix I).

## 7. STATISTICAL METHODOLOGY

### 7.1. Sample Size Estimate

The estimated sample size of 175 animals (35 per treatment group) is based on a previous study with the Principal Investigator, and will provide 80% power to declare a test formulation to be "at least as good as" a reference formulation with respect to total (whole mouth) enamel lesion (E) caries score. This calculation assumes that the coefficient of variation (c.v.) is 24% as a percentage of the reference (the c.v. observed in Study #936-9170), and that the underlying mean is not more than 5% higher for the experimental mouthrinse than for the positive control.

This sample size also provides approximately 80% power to declare two formulations equivalent, given the 24% c.v. and no more than a 5% difference between the underlying means.

### 7.2. Data Sets Analyzed

The primary analysis will include all animals entered into the study. In the unlikely event that animals die before the conclusion of treatment, they will be scored and included in the statistical analysis; a separate analysis will also be conducted without these animals, since it may reasonably be postulated that the shorter exposure to the challenge and treatment could unduly influence their caries response.

### 7.3. Primary and Secondary Efficacy Variables

The primary efficacy variable will be the total (whole mouth) enamel lesion (E) caries score. The secondary efficacy variables will be total (whole mouth) slight dentin (Ds) and moderate dentin (Dm) lesion caries scores; smooth surface E, Ds and Dm lesion caries scores, sulcal surface E, Ds and Dm lesion caries scores, and interproximal surface E, Ds and Dm lesion caries scores. See Appendix I for the details of Keyes Scoring Method.

### 7.4. Statistical Analysis

Data management and statistical analyses will be performed by the Statistics and Data Management Department at the Warner-Lambert Consumer Products Research & Development Division.

### 7.5. The primary questions

a Does experimental mouthrinse W2194-471 have anticaries efficacy?

b Does experimental mouthrinse W2194-471 have anticaries efficacy at least as good as that provided by positive control W2194-473

c Does the fixed combination of Listerine essential oils interfere with fluoride effectiveness or in itself have an anticaries effect?

### 7.6. The secondary questions

a. Is the study validated?

b. Does pH have an effect upon the anticaries activity of 0.02% sodium fluoride?

### 7.7. Success Criteria

To answer these questions, between-treatment comparisons will be performed using a mixed model, with treatment considered fixed and litter considered random.

*Experimental mouthrinse W2194-471 will be considered to have anticaries efficacy* if the mean post-treatment total (whole mouth) E lesion caries score for the experimental mouthrinse (W2194-471) is statistically significantly lower than the mean for both the negative controls (W10859-005 and W2194-396), based on two-sided tests of the null hypotheses that treatment means are different versus the alternative hypotheses that the means are different.

*Experimental mouthrinse W2194-471 will be considered to have anticaries efficacy at least as good as that of positive control* W2194-473 if +20% is above the upper limit of the one-sided 95% confidence interval for the difference (expressed as a percentage difference) between the means for the experimental mouthrinse (W2194-471) and positive control W2194-473. This procedure is a 0.05 level test of the null hypothesis that the mean for W2194-471 is at least 20% higher than the mean for the positive control, versus the alternative hypothesis that the mean for W2194-471 is less than 20% higher than the mean for the positive control.

*The study will be considered validated* if the mean post-treatment total (whole mouth) E lesion caries score for the positive control (W2194-473) is statistically significantly lower than the mean for the negative control (W10859-005), based on a two-sided test of the null hypothesis that the treatment means are equal versus the alternative hypothesis that the treatment means are different.

*To address whether the fixed combination of Listerine essential oils interfere with fluoride effectiveness or in itself have an anticaries effect*, it will be evaluated whether the experimental mouthrinse is equivalent in anticaries efficacy to the NaF Treatment Control. The experimental mouthrinse will be considered equivalent to the NaF Treatment Control if the two-sided 90% confidence interval for the difference (expressed as a percentage of the NaF Treatment Control) is within –20% to +20%. This procedure is equivalent to Schuirmann's two one-sided t-tests at the 0.05 level of significance. The null hypothesis is that the treatmetn means are at least 20% different as a percentage of the NaF Treatment Control, and the alternative hypothesis is that the treatment means are less than 20% different as a percentage of the NaF Treatment Control.

*To address whether pH has an effect upon anticaries efficacy, it will be evaluated whether the NaF Treatment Control is equivalent in anticaries efficacy to the positive control,* the NaF Treatment Control will be considered equivalent to the positive control if the two-sided 90% confidence interval for the difference (expressed as a percentage of the positive control) is within (and does not include) –20% to +20%. As noted above, this procedure is equivalent to Schuirmann's two one-sided t-tests at the 0.05 level of significance. The null hypothesis is that the treatment means are at least 20% different as a percentage of the positive control, and the alternative hypothesis is that the treatment means are less than 20% different as a percentage of the positive control.

Mouthrinse W2194-396 will be considered to have anticaries efficacy if the mean post-treatment total (whole mouth) E lesion caries score for mouthrinse W2194-396 is statistically significantly lower than the mean for the negative control (W10859-005), based on a two-sided test of the null hypothesis that the treatment means are equal versus the alternative hypothesis that the treatment means are different.

Since all tests implied by a single question must be consistent with stated outcomes to demonstrate the objective of that portion of the study, no multiple comparison penalties will be taken. All tests will be performed at the 0.05 level of significance.

The above analysis will also be performed for each secondary efficacy parameter.

Summary statistics will be provided for each treatment group.

### 7.8. Interim Analysis

No interim analyses will be performed.

### 7.9. Record Maintenance

All records (protocols, amendments, stratification, data sheets, and final reports) will be maintained in the University of Rochester archives. The hard tissue specimens will also be maintained in the Archives. Copies of all data sheets will be sent to Warner Lambert for analysis, and will be retained in the Statistics and Data Management study file.

## Appendix I. Keyes Scoring Method

The method divides the sulcal aspect of the mandibular molars into linear units: six for the first molar, four for the second, and four for the third molar. The severity scores are E, lesions present only in the enamel; $D_s$, lesions involving the DEJ; $D_m$, lesions extending into the dentin; and $D_x$, which represented breakdown of the dentin. The buccal involvement is obtained by determining the number of unit area in which caries has penetrated to the E, $D_s$, $D_m$, $D_x$ depth.

The estimation of the sulcal scores is achieved by applying a linear estimation to theoretically flattened-out sulci and evaluating depth as indicated previously for the buccal section. The number of linear units assigned to each sulcus beginning with the first to the third molars are: 1st mandibular molar 2, 3, 2; 2nd mandibular molar 3, 2; 3rd mandibular 2; 1st maxillary molar 2, 3; 2nd maxillary molar 3; 3rd maxillary molar 2. The number of linear units assigned to each molar as well as for the buccal-lingual surface are summarized in the following table.

| Lesion Type | Mandibular | | | Maxillary | | |
|---|---|---|---|---|---|---|
| | 1st | 2nd | 3rd | 1st | 2nd | 3rd |
| Buccal | 6 | 4 | 4 | 6 | 4 | 3 |
| Lingual | 6 | 4 | 4 | 6 | 4 | 3 |
| Sulcal | 7 | 5 | 2 | 6 | 3 | 2 |
| Proximal | 1 | 2 * | 1 | 1 | 2 * | 1 |

*One mesial and one distal unit

[a] Navia, Juan, N.: Animal Models in Dental Research, pp 287 and 290, 1977.

[b] Keyes, Paul H.: Dental Caries in the Molar Teeth of Rats. II. A Method for Diagnosing and Scoring Several Types of Lesions Simultaneously. Journal of Dental Research, pp 1088-1099, 1958.

## Appendix II. Random Code for Treatment Assignment

After distributing animals into balanced groups, have the animal handlers sequentially label the groups A-E. After groups are numbered, assign treatments to the groups according to the table below. Withhold this assignment code from the investigators applying treatment and the caries scorer.

| Product Code | Product # |
|---|---|
| | W10859-003 |
| | W2194-471 |
| | W2194-396 |
| | W2194-472 |
| | W2194-473 |

## Appendix III. Key to Treatments

**(Warner Lambert Use only. Remove from Investigator's Protocol)**

| Product Code | Product # | PRODUCT |
|---|---|---|
| | W10859-003 | Sterile Water |
| | W2194-471 | FreshBurst Listerine with 0.02% NaF (100 ppm $F^-$) |
| | W2194-396 | FreshBurst Listerine® |
| | W2194-472 | pH 4.2 NaF mouthrinse (100 ppm $F^-$) |
| | W2194-473 | Neutral 0.02% NaF mouthrinse (100 ppm $F^-$) |